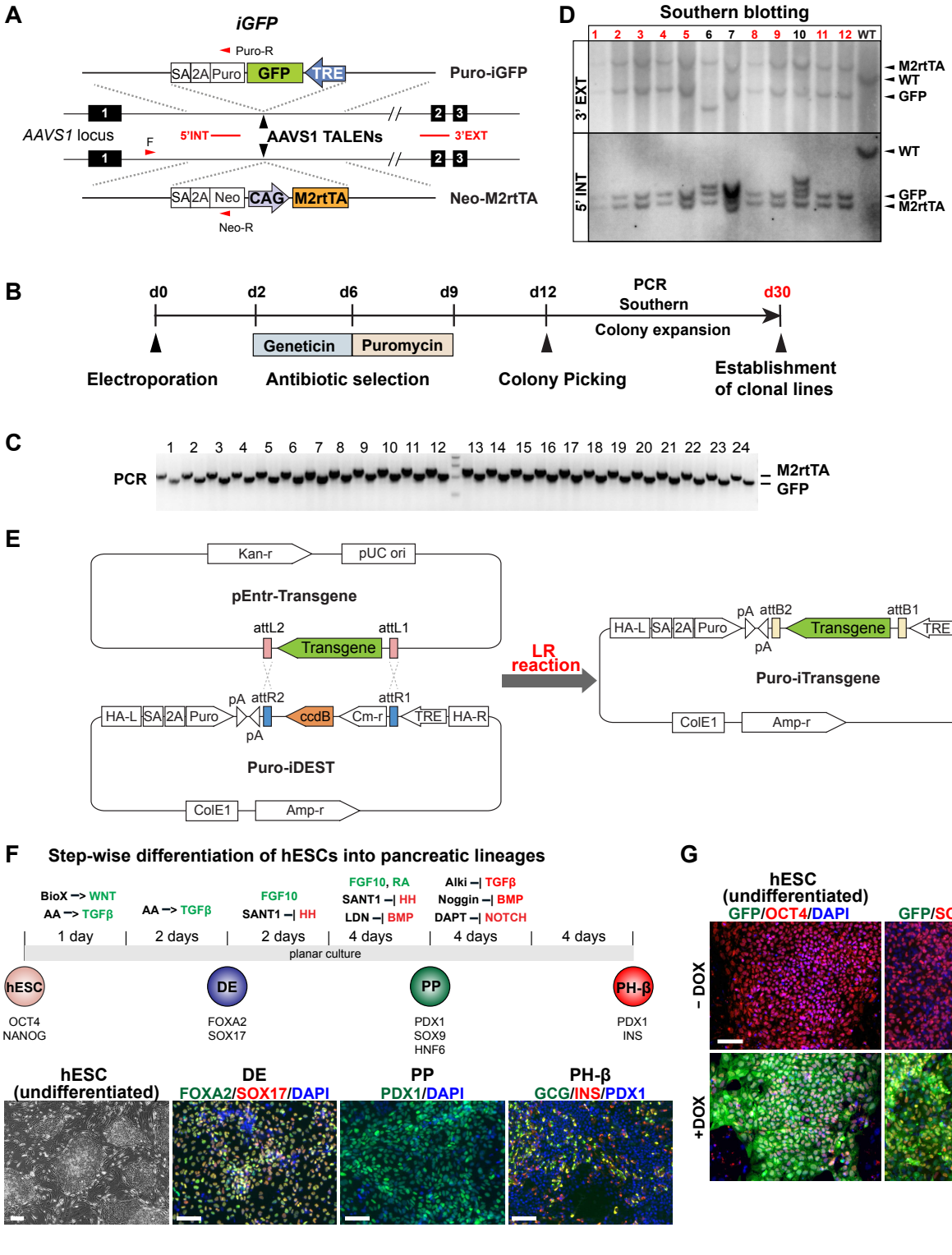
Supplemental Information

Inventory of Supplemental Information

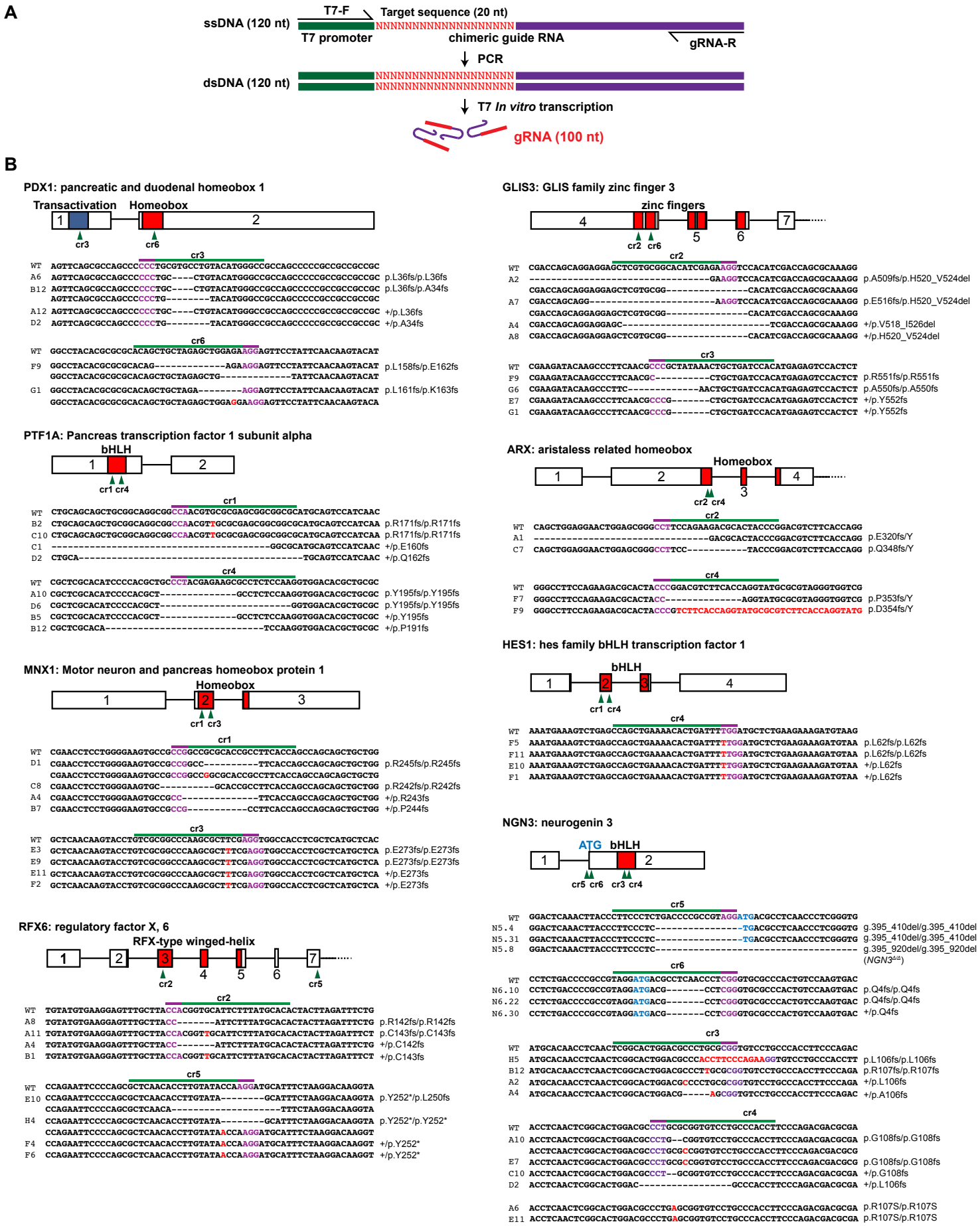
Zhu_Supplemental Figure 1



Supplemental Figure 1, related to Figure 1. TALEN-mediated gene targeting into the *AAVS1* locus for gain-of-function studies

A) Schematics for generating iGFP hESC lines for inducible GFP expression. Two primer pairs (F+Puro-R and F+Neo-R, indicated by red arrowheads, Table S4) were used for PCR screening of clones with correct insertions in both *AAVS1* alleles. Red lines indicate 3' external (3'EXT) and 5' internal (5'INT) probes used for Southern blotting analysis. **B)** Steps for establishment of clonal iGFP lines in ~1 month. **C)** PCR genotyping showed correct integration of GFP (F+Puro-R, 1,033bp) and M2rtTA (F+NeoR: 1,217 bp) into both *AAVS1* alleles in all hESC clones screened. **D)** Southern blotting analysis of iGFP hESC lines using 3' external and 5' internal probes. Lines indicated with red numbers carry correct insertions of the Puro-iGFP and Neo-M2rtTA donor sequences without random integrations. **E)** Schematics of the vectors and LR reaction to construct Puro-iTransgene donor plasmid using Gateway system. Recombination between the attL (attL1 and attL2) and attR (attR1 and attR2) sequences results in transfer of the transgene from pEntr-Transgene to Puro-iDEST to generate Puro-iTransgene. **F)** Schematics of the pancreatic differentiation protocol. Signaling pathways that are activated or inhibited during differentiation are highlighted in green or red respectively. BioX: GSK3 inhibitor BIO-acetoxime; AA: Activin A; HH: Hedgehog; DE: Definitive endoderm; PP: Pancreatic progenitor. Representative immunofluorescence staining images of cells at each differentiation stage were shown. **G)** Representative immunofluorescence staining for GFP and stage-specific markers in iGFP cells with or without doxycycline treatment. Cells were fixed and stained 48 hours after doxycycline (DOX) treatment. We generally detect transgene expression starting from 24 hours after doxycycline treatment. Scale bar = 100 μm in all figures unless otherwise indicated. (Related to Figure 1)

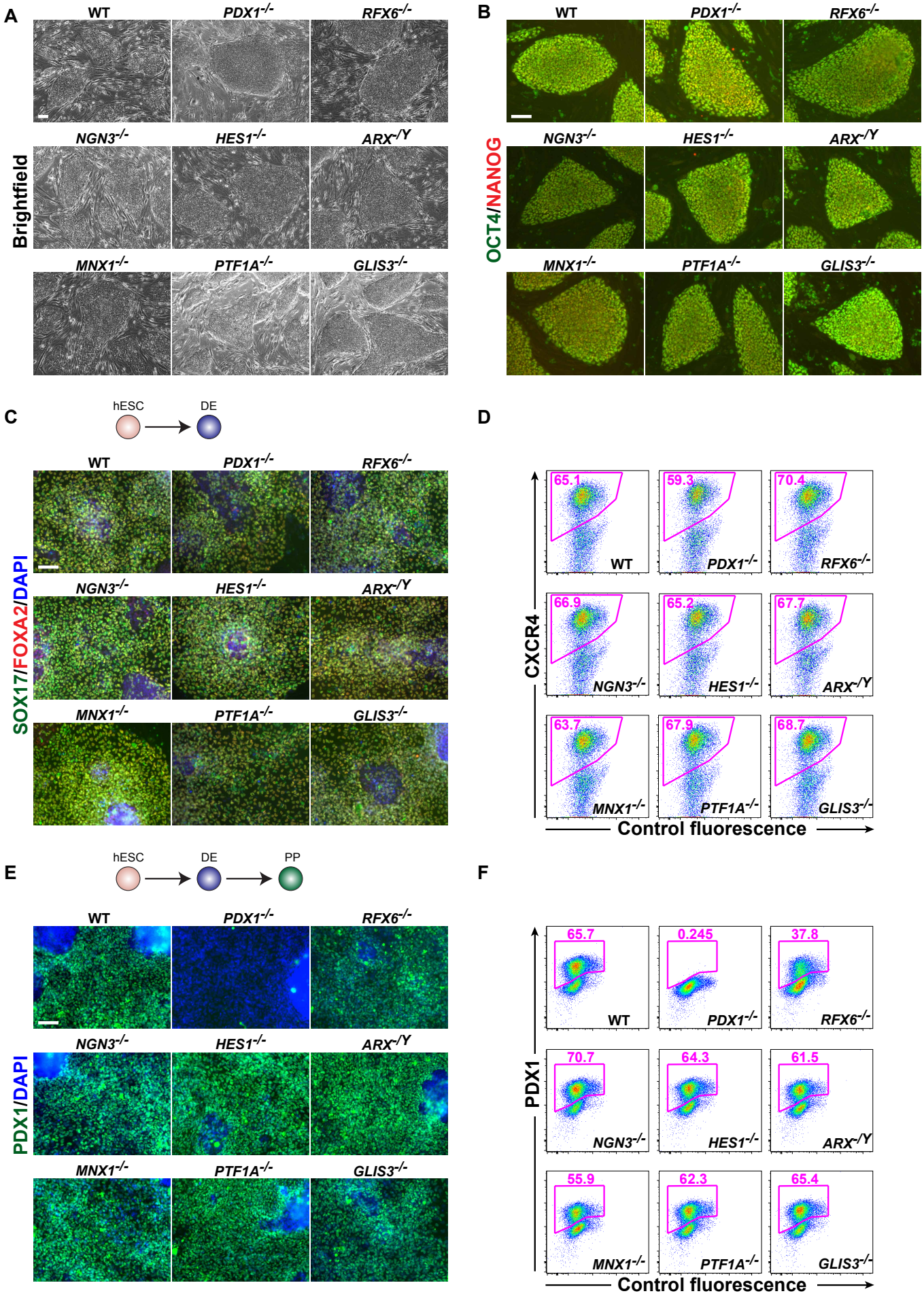
Zhu_Supplemental Figure 2



Supplemental Figure 2, related to Figure 2. Generation of hESC knockout lines

A) Illustration of PCR-based gRNA synthesis strategy. A 120-nt synthetic single strand DNA (ssDNA) containing the T7 promoter sequence, the 20-nt target sequence and the constant chimeric gRNA sequence is PCR amplified using the T7-F and gRNA-R primer pairs (Table S4). The PCR product is then used for gRNA production through T7 *in vitro* transcription. **B)** Schematics of gene targeting strategy for creating hESC knockout mutants for *PDX1, PTF1A, MNX1, NGN3, GLIS3, RFX6, ARX and HES1*. Exons and introns are represented by boxes and lines connecting the boxes respectively, and sequences corresponding to transactivation and DNA-binding domains are indicated in blue and red respectively. Positions of CRISPR target sites are indicated with arrowheads. Wild-type (WT) and mutant sequences adjacent to the target area are shown with green and purple colored bars (above the sequence) indicating the 20-nt gRNA target and the 5'-NGG-3' protospacer adjacent motif (PAM) sequences. Each dash in the mutant sequence indicates one base deletion and red colored bases indicate insertion or replacement. Mutant clone names are indicated on the left of the corresponding mutant DNA sequences, and the predicted protein changes are indicated on the right. (Related to Figure 2)

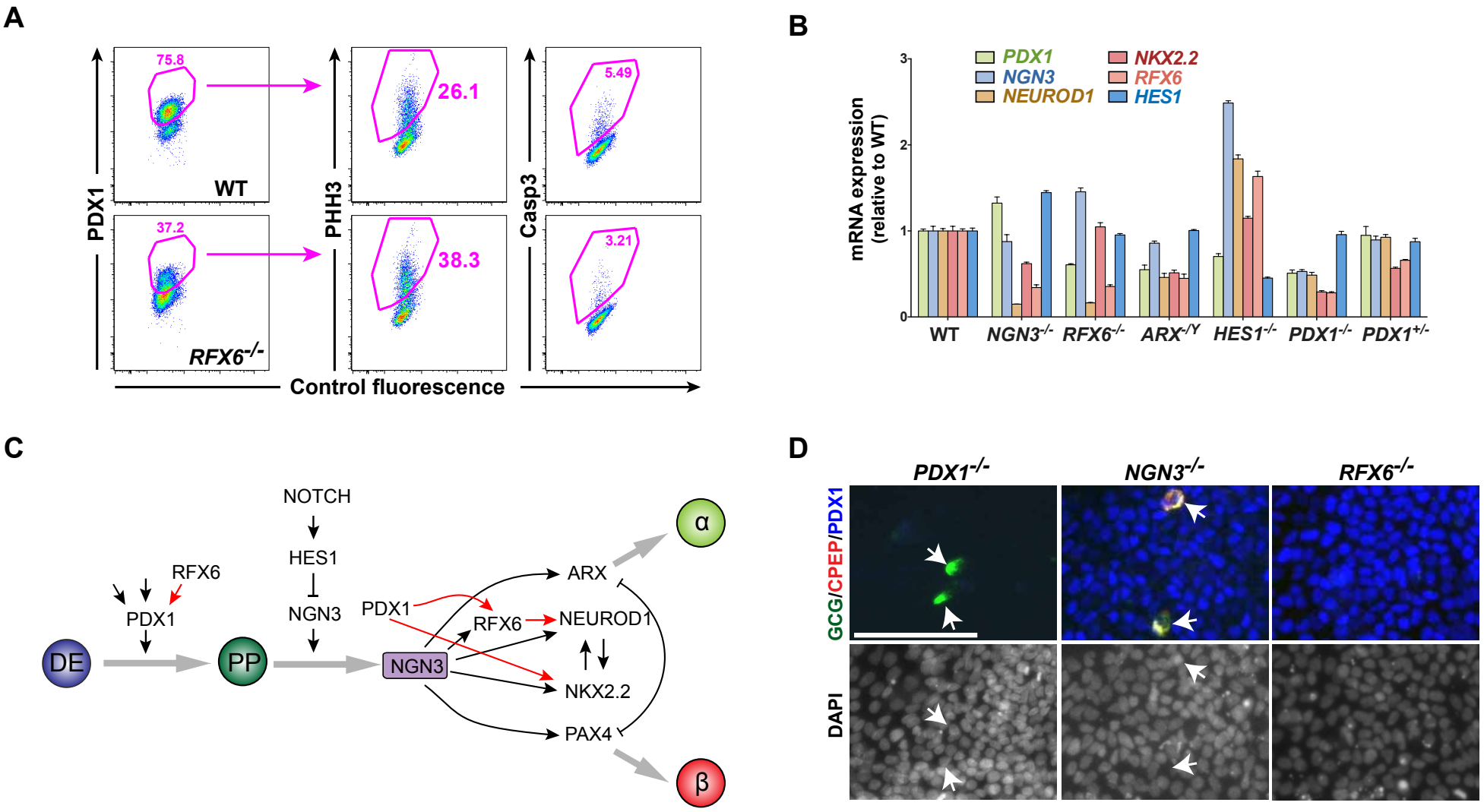
Zhu_Supplemental Figure 3



Supplemental Figure 3, related to Figure 3. Analysis of hESC mutant phenotypes

A, B) Representative brightfield images (**A**) and OCT4 and NANOG immunofluorescence staining (**B**) of undifferentiated wild-type control and knockout hESCs. **C, D)** Representative SOX17 and FOXA2 immunofluorescence staining (**C**) and FACS plots of CXCR4 expression (**D**) at the DE stage. **E, F)** Representative immunofluorescence staining (**E**) and FACS plots of PDX1 expression (**F**) at the PP stage. (Related to Figure 3)

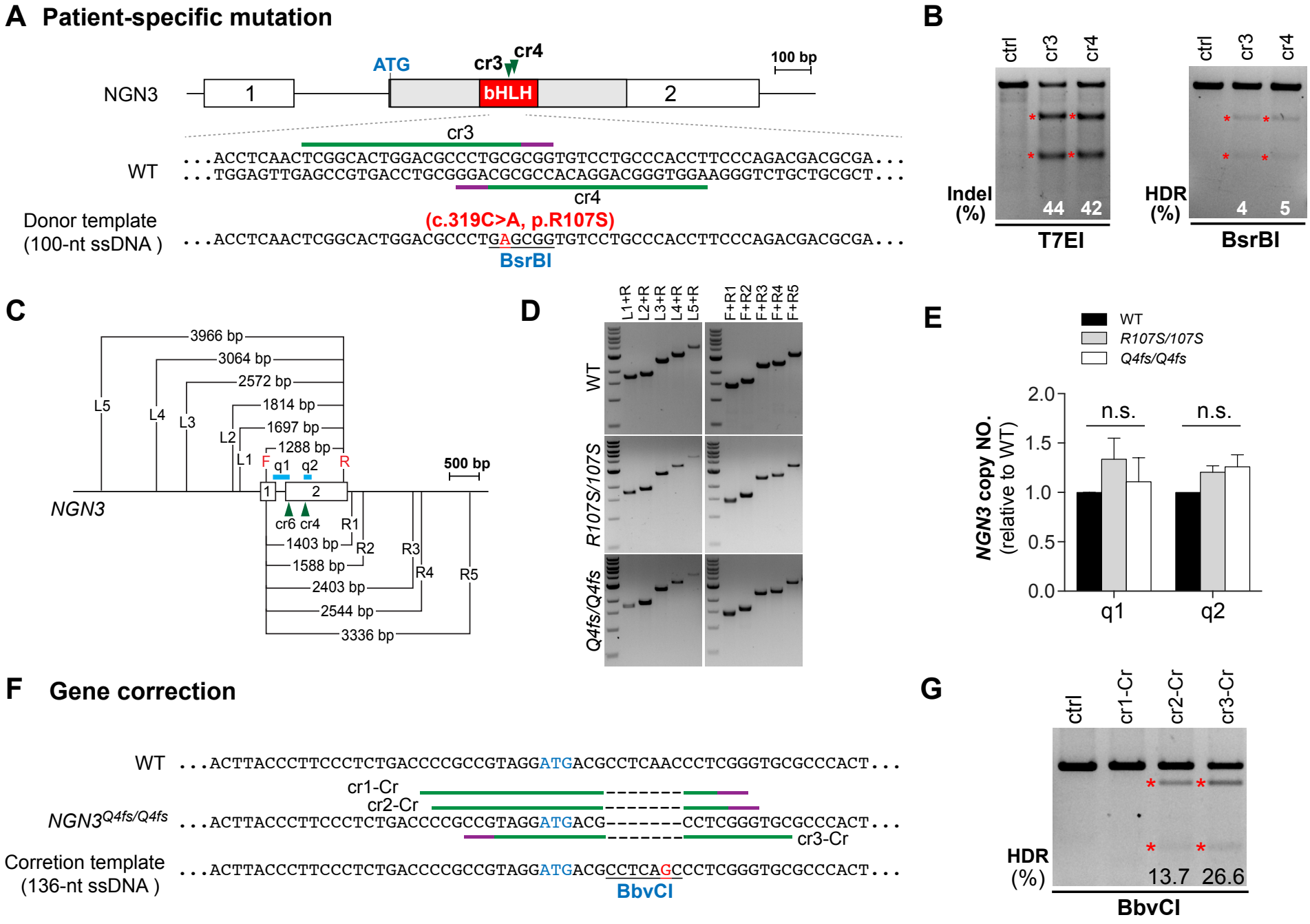
Zhu_Supplemental Figure 4



Supplemental Figure 4, related to Figure 4 and Figure 5. Further analysis of *RFX6*, *PDX1* and *NGN3* mutant hESC lines

A) Representative FACS plots of PHH3 and Casp3 expression in PDX1+ cells in wild-type and *RFX6*$^{-/-}$ mutants. PHH3: Phospho-Histone H3; Casp3: cleaved Caspas3. PHH3 expression was used to identify cells at the onset of mitosis (prophase, metaphase, and weaker expression at early anaphase). **B)** qRT-PCR analysis of pancreatic transcription factors in wild-type and hESC mutants at the PH-β cell stage. n = 4 to 8. **C)** Schematic diagram depicting a working model of gene regulatory network governing human pancreatic development. The lines indicate either direct or indirect gene regulation. Black lines indicate gene regulation uncovered from murine studies and conserved in human development as inferred from the present hESC-based study. Red lines indicate gene regulation revealed in our study but not previously reported, including human-specific regulations. **D)** Immunofluorescence staining for rare INS+ and/or GCG+ cells in *PDX1*$^{-/-}$ and *NGN3*$^{-/-}$ and *RFX6*$^{-/-}$ mutants. (Related to Figure 4 and Figure 5)

Zhu_Supplemental Figure 5

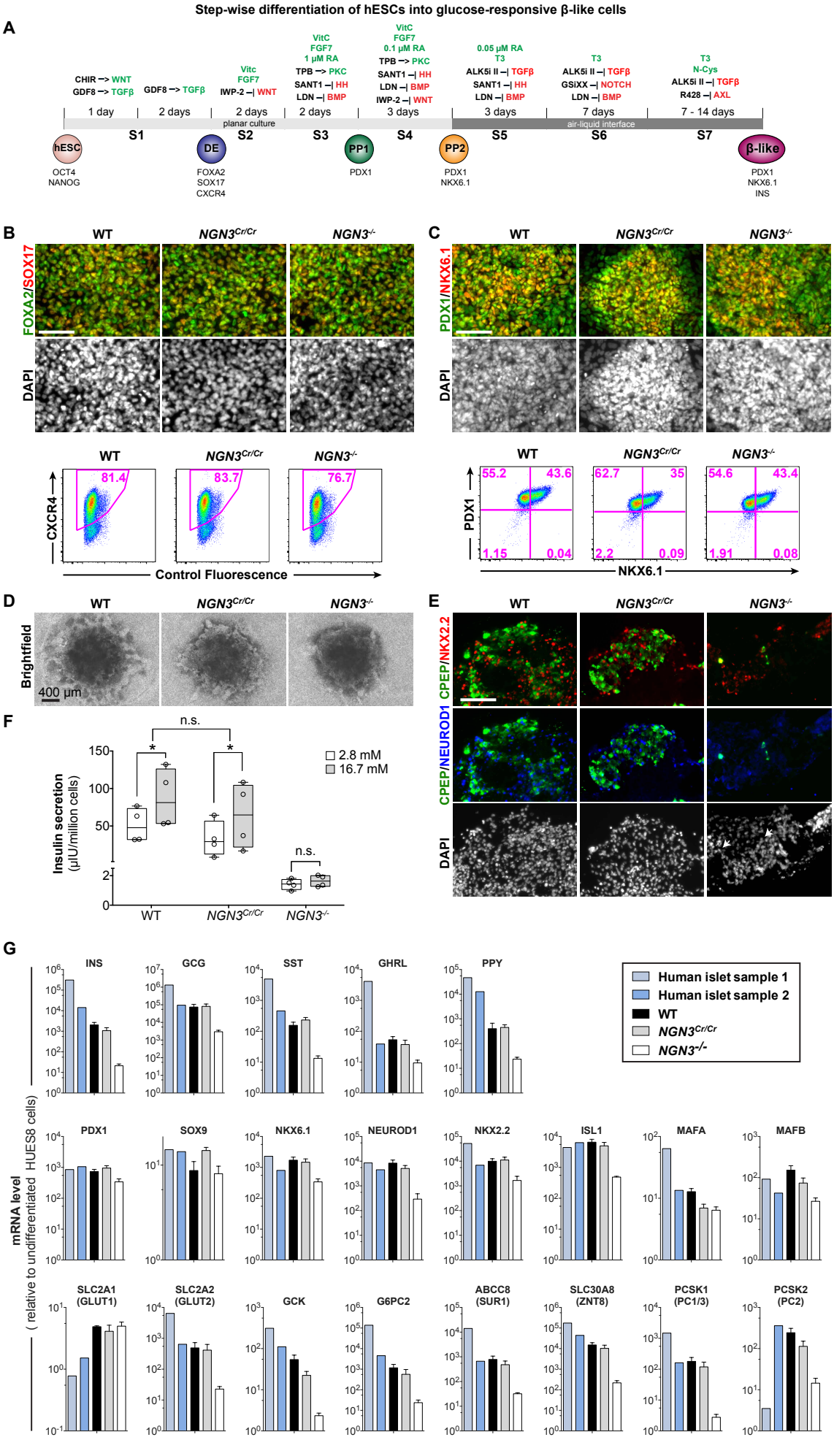


Supplemental Figure 5, related to Figure 5 and 6. The introduction of an *NGN3* patient-specific mutation and the correction of an *NGN3* Indel mutation

A) Schematics showing the generation of patient-specific *NGN3*$^{R107S/R107S}$ mutant lines. Two gRNAs, cr3 and cr4, were designed for gene editing. C>A substitution (red) was introduced using a 100-nt ssDNA HDR template, resulting in an R107S amino acid substitution. The C>A substitution also introduced a BsrBI restriction site that allows evaluation of the HDR efficiency through Restriction Fragment Length Polymorphism (RFLP). **B)** T7EI assay and BsrBI digestion for evaluating Indel and HDR efficiencies. Non-transfected wild-type hESCs were used as the control (Ctrl). Based on the results here, cr4 was chosen in subsequent experiments to create *NGN3*$^{R107S/R107S}$ lines. **C)** Schematics for detecting potential large deletions in homozygous *NGN3* mutant lines. We and others have found that CRISPR/Cas predominantly introduces small Indel mutations (< 50 bp), and the frequency of Indels appears to decrease exponentially with increasing Indel size (Bae et al., 2014; Yang et al., 2015). However, no systematic studies have been performed to determine the extent to which CRISPR/Cas could induce larger deletions. While Indels smaller than 500 bp are readily detectable by PCR amplification followed by gel electrophoresis, T7EI assay and Sanger sequencing, the PCR amplification method would miss deletions that disrupt one or both of the PCR primer binding sites. For instance, we identified homozygous mutant lines (e.g. *NGN3*$^{R107S/R107S}$) using a primer pair that amplified a 1,288 bp DNA fragment, but could there be a large deletion in one allele that extends beyond the boundaries of the PCR primers? To investigate this possibility, we extensively analyzed the *NGN3* genomic locus in two *NGN3* homozygous mutant lines (*NGN3*$^{R107S/R107S}$ and *NGN3*$^{Q4fs/Q4fs}$) using two complementary methods (long-range PCR and qPCR analysis of the genomic DNA) as illustrated here. Cr6 and cr4 (green triangle): gRNAs used to generate *NGN3*$^{R107S/R107S}$ and *NGN3*$^{Q4fs/Q4fs}$ mutant lines. F+R: the original PCR primers used for

T7EI assay and Sanger sequencing. Additional primers were indicated for amplifying genomic region up to ~4 kb on each side of the CRISPR targets. Multiple PCR primers were designed to increase the sensitivity of detection. q1 and q2 (blue bars): two amplicons of genomic DNA qPCR primers. **D)** Gel electrophoresis of PCR amplicons amplified using the indicated PCR primers on genomic DNA from wild-type, *NGN3*$^{R107S/R107S}$ and *NGN3*$^{Q4fs/Q4fs}$ mutants. In the 6kb genomic region scanned by these PCR primers, no large deletions were detected in either *NGN3*$^{R107S/R107S}$ or *NGN3*$^{Q4fs/Q4fs}$ mutant except for the 7-bp deletion already identified in *NGN3*$^{Q4fs/Q4fs}$ mutants using the original PCR primers (F+R). **E)** QPCR analysis of genomic DNA (q1, q2, indicated in panel C) from wild-type, *NGN3*$^{R107S/R107S}$ and *NGN3*$^{Q4fs/Q4fs}$ mutant lines. Results were normalized first to ACTB and then to wild-type cells from the same experiment (n=3). No significant differences were observed between *NGN3*$^{R107S/R107S}$, *NGN3*$^{Q4fs/Q4fs}$ mutants and wild-type cells. Thus these two mutant lines harbor the expected homozygous mutations, and there is no evidence suggesting that one allele of either mutant line carries a large deletion. **F)** Schematics showing the correction of an Indel mutation in the *NGN3*$^{Q4fs/Q4fs}$ mutant line (N6.10) back into wild-type sequence. Three gRNAs, cr1, cr2 and cr3, were designed for targeting mutant DNA. A 136-nt ssDNA was used as the HDR template for gene correction. The HDR template also contains a silent mutation (A>G) that introduces a BbvCI restriction site without affecting the wild-type protein sequence. **G)** An RFLP assay based on BbvCI digestion was used to determine the gene correction efficiency, and the most efficient gRNA (cr3-Cr) was chosen to generate the *NGN3*$^{Cr/Cr}$ lines. Non-transfected *NGN3*$^{Q4fs/Q4fs}$ hESCs were used as the control (Ctrl). (Related to Figure 5 and Figure 6)

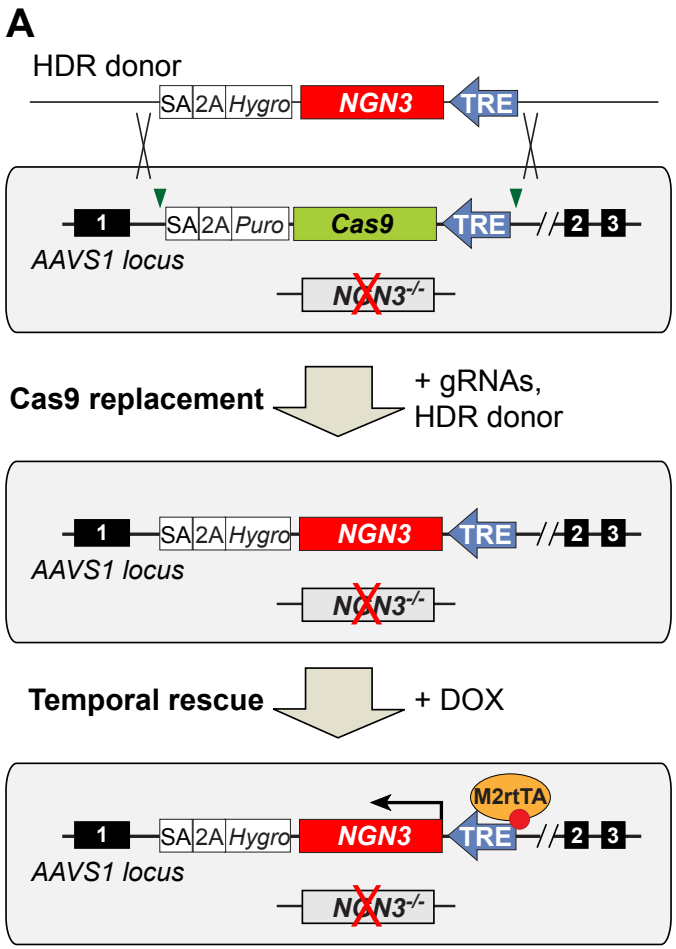
Zhu_Supplemental Figure 6

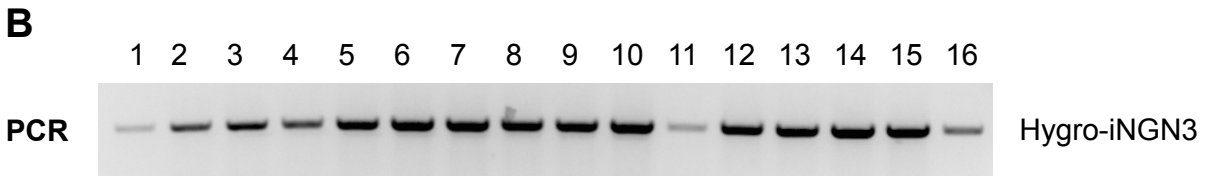


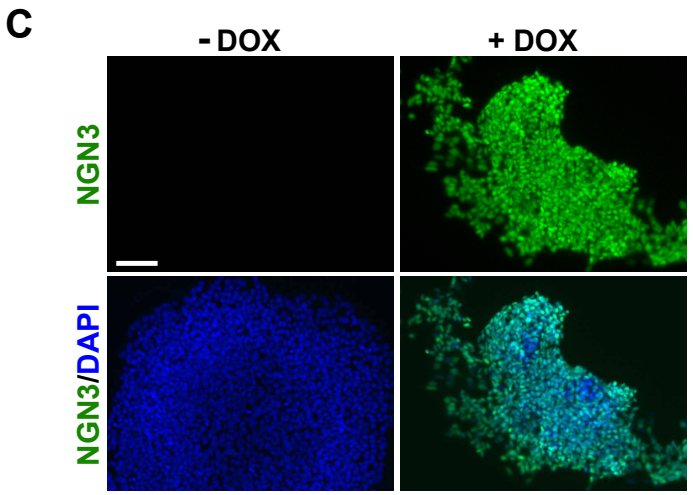
Supplemental Figure 6, related to Figure 6. The requirements for *NGN3* in the formation of glucose-responsive β-like cells

A) Schematics showing the step-wise differentiation of hESCs into glucose responsive β-like cells. Signaling pathways that are activated or inhibited during differentiation are highlighted in green or red respectively. CHIR: GSK3 inhibitor; PP1: PDX1+ early pancreatic progenitor; PP2: PDX1+NKX6.1+ pancreatic progenitor. **B)** Representative immunofluorescence staining of SOX17 and FOXA2 expression and FACS plots of CXCR4 expression in wild-type, $NGN3^{Cr/Cr}$ and $NGN3^{-/-}$ cells at DE stage. **C)** Representative immunofluorescence staining and FACS plots of PDX1 and NKX6.1 expression in wild-type, $NGN3^{Cr/Cr}$ and $NGN3^{-/-}$ cells at PP2 stage. **D)** Representative morphology of cell aggregate culture in air-liquid interface for wild-type, $NGN3^{Cr/Cr}$ and $NGN3^{-/-}$ cells at the β-like cell stage (d34). **E)** Representative immunofluorescence staining of NEUROD1 and NKX2.2 expression in wild-type, $NGN3^{Cr/Cr}$ and $NGN3^{-/-}$ cells at the β-like cell stage. **F)** Insulin secretion assay for wild-type, $NGN3^{Cr/Cr}$ and $NGN3^{-/-}$ cells at the β-like cell stage. As the high and low glucose treatments were performed sequentially on the same cells, results were analyzed using two-tailed paired student t-test. The asterisk indicates $P < 0.05$. **G)** qRT-PCR analysis of the expression of pancreatic hormones, transcription factors essential for β cell identity and function, and cellular components for GSIS in human islets, wild-type, $NGN3^{Cr/Cr}$ and $NGN3^{-/-}$ cells at the β-like cell stage. (Related to Figure 6)

Zhu_Supplemental Figure 7







Supplemental Figure 7, related to Figure 7. Temporal regulation of NGN3 activity in the *NGN3*$^{-/-}$ mutant background

A) Schematics showing cassette exchange of Puro-iCas9 with Hygro-iNGN3 in *NGN3*$^{-/-}$ mutant background. A pair of gRNAs (green triangle) was designed for co-transfection with the Hygro-iNGN3 HDR donor plasmid. Successful cassette exchange should allow temporal control of *NGN3* expression by doxycycline treatment. F+Hygro-R: PCR genotyping primers (Table S4), **B)** PCR detection of correctly targeted clones. **C)** Immunofluorescence staining showing the induction of NGN3 expression with doxycycline treatment. (Related to Figure 7)

Supplemental Experimental Procedures

Maintenance of hESCs

All experiments were performed on HUES8, a well-characterized hESC line (NIH approval number: NIHhESC-09-0021) for pancreatic differentiation. HUES8 hESCs (18 - 50 passages) were cultured on irradiated mouse embryonic fibroblasts (iMEFs) feeder layers in DMEM/F12 (without HEPES) supplemented with 20% KnockOut Serum Replacement, 1X Non-Essential Amino Acids, 1X GlutaMAX, 100 U/mL Penicillin/100 μg/mL Streptomycin (Gemini), 0.055 mM 2-mercaptoethanol and 10 ng/mL recombinant human basic FGF. Cultures were passaged at a 1:6−1:12 split ratio every 4−6 days using 0.05% trypsin/EDTA. 5 μM Rho-associated protein kinase (ROCK) inhibitor Y-27632 (Selleck Chemicals, S1049) was added into culture media when passaging or thawing cells. For differentiation into glucose-responsive β-like cells, hESCs were adapted to the E8 media condition following manufacturer's instructions and cultured on Vitronectin (VTN)-coated plates. E8-adapted hESCs were dissociated with 0.5 mM EDTA, and passaged at a 1:10−1:15 split ratio every 4−6 days for maintenance. All cell culture reagents were purchased from Life Technologies unless otherwise indicated. We routinely test hESCs in culture to ensure that the cells are free of mycoplasma contamination. All hESC work was conducted according to NIH guidelines and approved by the Embryonic Stem Cell Research Committee (ESCRO).

AAVS1 targeting vectors

A pair of TALENs (AAVS1-TALEN-L and AAVS1-TALEN-R; Addgene 59025 and 59026) was used to target the first intron of the constitutively expressed gene *PPP1R12C* at the *AAVS1* locus as previously described (Gonzalez et al., 2014). The Puro-iDEST plasmid was constructed by replacing the EGFP sequence in the TRE-TIGHT-EGFP-BW plasmid

(Addgene 22077) with a Gateway destination cassette (Life Technologies, Gateway® system). For targeting the *AAVS1* locus for inducible gene expression, the gene of interest ("transgene") was first cloned into the pENTR vector, creating the pENTR-transgene plasmid. Next using the pENTR-transgene and Puro-iDEST plasmids, the *AAVS1* targeting plasmid Puro-iTransgene was generated through the Gateway LR reaction (Figure S1E). For inducible Notch signaling activation and NGN3 forced expression, mouse Notch1 intracellular domain, the constitutively active form of *Notch* (Addgene: 15079) and mouse *Ngn3* cDNA were used to generate the Puro-iNotchIC and Puro-iNGN3 constructs respectively following the aforementioned cloning strategy.

For temporal control of *NGN3* activity in *NGN3*$^{-/-}$ hESCs, the Hygro-iDEST plasmid was first constructed by sequential cloning of the *AAVS1* homology arms, the PCR amplified SA-2A-Hygro cassette and the TRE-iDEST cassette (amplified from the Puro-iDEST plasmid) into the pBlueScript SKII (+) backbone. Next, wild-type *NGN3* coding sequence was PCR amplified from HUES8 genomic DNA and cloned into pENTR vector to generate pENTR-NGN3 plasmid. Finally, the Hygro-iNGN3 plasmid was generated through Gateway LR reaction between pENTR-NGN3 and Hygro-iDEST plasmids.

Electroporation for establishment of inducible gene expression lines

hESCs were pre-treated with ROCK inhibitor for 24 hours before plasmid electroporation. On the day of electroporation, hESCs were dissociated into single cells with 0.25% Trypsin/EDTA and filtered through a 40 μm cell strainer to remove cell clumps. 10 million cells were resuspended in 800 μL cold PBS and mixed with four plasmids (5 μg AAVS1-TALEN-L, 5 μg AAVS1-TALEN-R, 40 μg Puro-iTransgene and 40 μg Neo-M2rtTA) for 5 min. Cells were then electroporated using Gene Pulser XceII (Bio-Rad) at 250 V, 500 μF in a 0.4 cm Gene Pulser cuvette (Bio-Rad), and replated on DR4

iMEFs (ATCC). 2 days after electroporation, cells were treated with 50 μg/mL of Geneticin for 4 days, followed by treatment with 0.5 μg/mL of Puromycin for another 3 days. After antibiotic selection, 6−12 colonies were picked based on hESC morphology, mechanically disaggregated and replated into individual wells of 24-well plates. Colonies were allowed to grow to near confluence and split and replica-plated in 6-well plates. Once confluent one replica was used for genomic DNA extraction and Southern blot analysis and the other for frozen stocks.

Southern blot analysis

To identify correctly targeted hESC iEXPRESS lines, genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen), which was used for all procedures involving genomic DNA isolation in this study. 5−10 μg of genomic DNA was digested overnight with 20U of SphI and subjected to electrophoresis in 1% agarose gels. The gels were denatured, neutralized, and transferred overnight by capillarity onto Hybond-N membranes (GE Healthcare) using the 10X SSC transfer buffer. Hybridization with 3' external or 5' internal probe was carried out overnight at 65 °C. The 3' external and 5' internal probes were generated by PCR on plasmid templates using the PCR DIG Probe Synthesis Kit (Roche) and the following primers: 3'F (ACAGGTACCATGTGGGGTTC) and 3'R (CTTGCCTCACCTGGCGATAT) for the 3' probe; 5'F (AGGTTCCGTCTTCCTCCACT) and 5'R (GTCCAGGCAAAGAAAGCAAG) for the 5' probe. The 3' external probe plasmid template was a gift from D. Hockemeyer. For the 5' internal probe, we used the Neo-M2rtTA donor as a template. For membrane hybridization, 5 μL of denatured DIG-labeled PCR product were used in 20 mL hybridization buffer. Probes were detected using an alkaline phosphatase-conjugated digoxigenin antibody (Roche) with CDP-Star (Roche) as a substrate for chemiluminescence.

gRNA design and synthesis

For each gene in this study, 4 to 6 gRNAs were designed using the online CRISPR design tool from Feng Zhang's laboratory (http://crispr.mit.edu/) (Hsu et al., 2013), and 2 gRNAs with the highest mutagenic efficiencies based on the T7EI assay were used for the generation of mutant lines. The T7EI assay is described in more details in previous studies (Gonzalez et al., 2014; Zhu et al., 2014).

For PCR-based gRNA synthesis, a 120-nt synthetic ssDNA containing the T7 promoter sequence followed by the variable 20-nt gRNA target sequence and the remaining constant gRNA sequence (Table S4) was first ordered from Integrated DNA Technologies. We then used 1 µl of the 25 nM ssDNA diluted in ddH2O and a pair of universal primers (T7-F and gRNA-R, Table S4) in a 50 µl PCR reaction to generate the double-stranded DNA (dsDNA) template for gRNA production through *in vitro* transcription using the MEGAshortscript T7 kit (Life Technologies). The resulting gRNAs were purified using the MEGAclear kit (Life Technologies), eluted in RNase-free water and stored at -80°C until use.

Glucose stimulated insulin secretion

Glucose stimulated insulin secretion assay was performed following previously described protocols (Kroon et al., 2008; Pagliuca et al., 2014; Rezania et al., 2014). In brief, ~4−6 S7 cell aggregates were transferred using wide orifice pipet tip into an Eppendorf tube and rinsed three times with the KRBH buffer (129 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 5 mM NaHCO3, 10 mM HEPES, 0.1% BSA in ddH2O and sterile filtered). Cell aggregates were pre-incubated in KRBH at 37°C for 1 hour to remove residual insulin from the culture medium. Cell aggregates were then incubated in KRBH spiked with 2.8 mM Glucose at 37°C for 30 min and

supernatants were collected. Next, cell aggregates were rinsed three times with KRBH, incubated in KRBH spiked with 16.7 mM Glucose at 37°C for 30 min and supernatants were collected again. At the end of the experiment, cell aggregates were dissociated into single cells using TrypLE Select, and the cell numbers were counted. Ultrasensitive Insulin ELISA kit (Alpco) was used to measure the insulin content in supernatant samples following manufacturer's instruction.

Establishment of clonal hESC mutant lines

The generation and transfection of HUES8 iCas9 hESCs was described previously (Gonzalez et al., 2014; Zhu et al., 2014). For generating mutant lines, iCas9 hESCs were treated with doxycycline for 1−2 days before gRNA transfection. Cells were dissociated using Accutase (Stem Cell Technologies) or TrypLE Select, replated onto iMEF-coated plates and transfected in suspension with gRNA (and ssDNA for HDR) using Lipofectamine RNAiMAX (Life Technologies) following manufacturer's instructions. gRNA and ssDNA were added at a 10 nM and 20 nM final concentrations respectively, unless otherwise indicated. gRNAs (or gRNA+ssDNA) and Lipofectamine RNAiMAX were diluted separately in Opti-MEM, mixed together, incubated for 5 min at room temperature (RT), and added dropwise to cultured hESCs. A second transfection was performed 24 hours later in some experiments.

Two days after the last gRNA/ssDNA transfection, hESCs were dissociated into single cells and replated at a low density (2,000 cells/10cm dish). Cells were allowed to grow until colonies from single cells became visible (~10 days). 36~48 colonies were picked based on hESC morphology, mechanically disaggregated and replated into individual wells of 96-well plates. Clonal lines were expanded and analyzed by Sanger sequencing to identify mutant clones. PCR and sequencing primers are provided in Table S4. Clonal

hESC lines carrying desired mutations were further expanded and frozen down. For PDX1 heterozygous mutants and all compound heterozygous mutants, sub-cloning of cells and TA cloning of PCR products were performed to exclude any contamination of cells with different genotypes.

Temporal control of *NGN3* activity in *NGN3*$^{-/-}$ hESCs

To generate *Hygro-iNGN3;NGN3*$^{-/-}$ cells for temporal control of *NGN3* expression, *Puro-iCas9;NGN3*$^{-/-}$ cells were treated with doxycycline for two days before transfection. Cells were dissociated using TrypLE Select into single cells, replated onto VTN-coated plates and transfected in suspension with a pair of gRNAs (AAVS1-cr1-ex and cr2-ex, Table S4) and the Hygro-iNGN3 donor plasmid using Lipofectamine 3000 (Life Technologies) following manufacturer's instructions. The gRNA and plasmid were added at 10 nM and 5 μg/ml final concentrations respectively and no doxycycline was added on the day of transfection. Media were changed 24 hours after transfection. Cells were allowed to grow until ~80% confluency, and then replated into 10-cm culture plates and subject to Hygromycin selection for 4 days. After antibiotic selection, 6 to 12 colonies were picked and expanded as previously described.

Quantitative RT-PCR

Total RNA was isolated with the RNeasy Mini Kit (Qiagen). DNA was removed from RNA samples using genomic DNA Eliminator spin columns. cDNA was produced using ~1 μg of total RNA using SuperScript III First-Strand Synthesis System (Life Technologies). Quantitative real-time PCR was performed in triplicate using ABsolute QPCR SYBR Green Low ROX Mix (Thermo Scientific). The primers used for quantitative RT-PCR were listed in Table S4.

Immunofluorescence staining

For immunofluorescence staining, cells were fixed in 4% paraformaldehyde for 10 min at RT, washed once with PBS and permeabilized in PBS with 0.1% Triton (PBST) for 15 min. Blocking was done for 5 min at RT with blocking solution (5% donkey serum in PBST). Primary and second antibodies were diluted in blocking solution. Primary antibodies were incubated at RT for 1 hour or overnight at 4 °C, and secondary antibodies at RT for 1 hour. The following primary antibodies and dilutions were used: rabbit anti-FOXA2, 1:200 (Millipore 07-633); goat anti-SOX17, 1:500 (R&D AF1924); goat anti-PDX1, 1:500 (R&D AF2419); mouse anti-NKX6.1, 1:500 (DSHB F55A12); mouse anti-NKX2.2, 1:100 (DSHB 74.5A5); goat anti-NEUROD1, 1:100 (Santa Cruz sc-1084); mouse anti-C-Peptide, 1:2,000 (Millipore 05-1109); rat anti-C-Peptide, 1:2,000 (DSHB GN-ID4-c); guinea pig anti-Insulin, 1:2,000 (Dako A0564); guinea pig anti-Glucagon, 1:2,000 (LINCO 4031-01F); mouse anti-Glucagon, 1: 1,000 (Sigma G2654); goat anti-SOX2, 1:100 (Santa Cruz sc-17320); Goat anti-OCT4, 1:100 (Santa Cruz sc-8628); rabbit anti-NANOG, 1:100 (Cosmobio Japan REC-RCAB0004P-F).

For cryosectioning, cell aggregates were collected from air-liquid interface culture at the β-like cell stage, and rinsed with PBS followed by overnight fixation in 4% PFA at 4 °C. Following fixation, PFA was removed and cells were rinsed three times with PBS and incubated overnight at 4 °C in 30% sucrose solution. The samples were next overlaid with OCT solution and frozen using dry ice and stored at −80 °C. Cryostat was used to cut 10 µm sections and placed on Superfrost plus slides. The sections were next rinsed with PBS and permeabilized with 0.1% PBST for 30 min, rinsed again with PBS and then blocked with appropriate serum for 30 min at RT. Primary antibodies were added at appropriate dilutions overnight at 4 °C. Secondary antibodies with DAPI were added for

1 hour at RT followed by rinsing with PBS and mounted using Fluoromout-G (Southern Biotech).

Western Blotting

Protein samples were collected from cell lysate homogenized in RIPA buffer supplemented with proteinase inhibitor cocktail (Cell Signaling Technology) and stored in −80ºC until use. After denatured and reduced in NuPAGE LDS sample buffer (Life technologies) and sample reducing agent (Life Technologies), protein samples were subject to gel electrophoresis in NuPAGE Bis-Tris Precast Gels and transferred to nitrocellulose membrane. Membranes were blocked in TBST buffer with 5% milk for 1 hour at RT and then incubated with primary antibodies overnight at 4ºC and HRP-conjugated secondary antibodies for 1 hour at RT. ECL western blotting substrate (Pierce) were used to visualize the protein bands. The following primary antibodies and dilutions were used: goat anti-PDX1 (recognizing a.a. 91-283, which includes the homeodomain), 1:1,000 (R&D AF2419); mouse anti-MNX1, 1:1,000 (DSHB 81.5C10-c, recognizing the C-terminus); rabbit anti-HES1, 1:1,000 (Santa Cruz, SC25392, recognizing a.a. 163-194).

Flow cytometry

Cells were dissociated into single cells using TrypLE Select and re-suspended in FACS buffer (5% FBS in PBS) at ~1X10^6 cells/100 µl. Cells were incubated with LIVE⁄DEAD Fixable Violet Dead Cell Stain Kit (Molecular Probes L34955, 1:1,000) for 30 min on ice for discrimination of live/dead cells. For cell surface markers staining, antibodies were also added at this time. After incubation, cells were rinsed twice and re-suspended in FACS buffer for analysis. For intracellular staining, after incubation with LIVE/DEAD dye and cell surface markers, cells were fixed and permeabilized using Foxp3 Staining

Buffer Set (eBioscience) for 1 hour at RT following manufacturer's instruction. For conjugated primary antibody, cells were incubated for 30 min at RT, washed twice and re-suspended in FACS buffer for analysis. For non-conjugated primary antibody, cells were first incubated with primary antibody for 30 min at RT, washed twice, incubated with fluorescence-conjugated secondary antibody for 30 min at RT, washed twice and re-suspended in FACS buffer for analysis. The following primary antibodies and dilutions were used: CXCR4-APC, 1:25 (R&D FAB170A); SOX17-PE, 1:50 (BD Biosciences 561591); goat anti-PDX1, 1:250 (R&D AF2419); mouse anti-NKX6.1, 1:250 (DSHB F55A12); rat anti-C-peptide, 1:500 (DSHB GN-ID4-c); mouse anti-GCG, 1:250 (Sigma G2654); rabbit anti-SST, 1:250 (DAKO A0566).

Supplemental Table 1, related to Figure 2. Establishment of clonal hESC mutant lines.

Gene	CRISPR	Clonal mutant type			
		Indel / Indel		+ / Indel	
PDX1	PDX1-cr3	24.4%	(10/41)	36.6%	(15/41)
	PDX1-cr6	48.8%	(21/43)	0%	(0/43)
PTF1A	PTF1A-cr1	18.2%	(8/44)	11.4%	(5/44)
	PTF1A-cr4	35.9%	(14/39)	17.9%	(7/39)
GLIS3	GLIS3-cr2	33.3%	(15/45)	40.0%	(18/45)
	GLIS3-cr6	21.7%	(10/46)	21.7%	(10/46)
RFX6	RFX6-cr2	17.0%	(8/47)	27.6%	(13/47)
	RFX6-cr5	20.4%	(9/44)	13.6%	(6/44)
MNX1	MNX1-cr1	26.1%	(12/46)	19.6%	(9/46)
	MNX1-cr3	60.4%	(29/48)	12.5%	(6/48)
ARX	ARX-cr2	35.7%	(15/42)[#]	N/A	
	ARX-cr4	34.1%	(14/41)[#]	N/A	
HES1	HES1-cr1	2.6%	(1/39)	10.2%	(4/39)
	HES1-cr4	4.4%	(2/45)	17.8%	(8/45)
NGN3	NGN3-cr5	25.0%	(12/48)	4.2%	(2/48)
	NGN3-cr6	16.7%	(6/36)	2.8%	(1/36)

This table indicates the percentage of monoallelic (Indel/+) and biallelic (Indel/Indel) mutant lines identified for each gene targeted. The numbers in parentheses indicate the number of monoallelic and biallelic lines in relation to the total number of lines screened in each experiment.

[#]: *ARX* is on the X chromosome. The percentage indicates cells with the Indel/Y genotype as the HUES8 line used in this study was from a male donor (Cowan et al., 2004).

Supplemental Table 2, related to Figure 5. Establishment of clonal hESC mutant lines carrying the patient-specific *R107S/R107S* mutation.

Gene	CRISPR	Clonal mutant type					
		+ /+	Indel / +	Indel / Indel	R107S / +	R107S / R107S	R107S / Indel
NGN3	NGN3-cr3	60.6% (57/94)	8.51% (8/94)	23.4% (22/94)	3.19% (3/94)	1.06% (1/94)	3.19% (3/94)
	NGN3-cr4	37.1% (33/89)	34.8% (31/89)	18.0% (16/89)	2.25% (2/89)	3.37% (3/89)	4.49% (4/89)

This table indicates the percentage of mutant lines identified for *NGN3* in different categories. The numbers in parentheses indicate the number of specific types of mutant lines in relation to the total number of lines screened in each experiment.

Supplemental Table 3, related to Figure 2. A summary of hESC mutant lines investigated in this study.

Gene	Biallelic mutant hESC lines			Monoallelic mutant hESC lines			CRISPR
	Number of lines	Clone name	Mutant description	Number of lines	Clone name	Mutant description	
PDX1	**4**	A6	p.L36fs/p.L36fs	**2**	A12	+/p.L36fs	PDX1-cr3
		B12	p.L36fs/p.A34fs		D2	+/p.A34fs	
		F9	p.L158fs/p.E162fs				PDX1-cr6
		G1	p.L161fs/p.K163fs				
PTF1A	**4**	B2	p.R171fs/p.R171fs	**4**	C1	+/p.E160fs	PTF1A-cr1
		C10	p.R171fs/p.R171fs		D2	+/p.Q162fs	
		A10	p.Y195fs/p.Y195fs		B5	+/p.Y195fs	PTF1A-cr4
		D6	p.Y195fs/p.Y195fs		B12	+/p.P191fs	
GLIS3	**4**	A2	p.A509fs/p.H520_V524del	**4**	A4	+/p.V518_I526del	GLIS3-cr2
		A7	p.E516fs/p.H520_V524del		A8	+/p.H520_V524del	
		F9	p.R551fs/p.R551fs		E7	+/p.Y552fs	GLIS3-cr6
		G6	p.A550fs/p.A550fs		G1	+/p.Y552fs	
MNX1	**4**	D1	p.R245fs/p.R245fs	**4**	A4	+/p.R243fs	MNX1-cr1
		C8	p.R242fs/p.R242fs		A7	+/p.P244fs	
		E3	p.E273fs/p.E273fs		E11	+/p.E273fs	MNX1-cr3
		E9	p.E273fs/p.E273fs		F2	+/p.E273fs	
RFX6	**4**	A8	p.R142fs/p.R142fs	**4**	A4	+/p.C142fs	RFX6-cr2
		A11	p.C143fs/p.C143fs		B1	+/p.C143fs	
		E10	p.Y252*/p.L250fs		F4	+/p.Y252*	RFX6-cr5
		H4	p.Y252*/p.Y252*		F6	+/p.Y252*	
ARX[#]	**4**	A1	p.E320fs/Y	**NA**			ARX-cr2
		C7	p.Q348fs /Y				
		F7	p.P353fs/Y				ARX-cr4
		F9	p.D354fs/Y				
HES1	**2**	F5	p.L62fs/p.L62fs	**2**	E10	+/p.L62fs	HES1-cr4
		F11	p.L62fs/p.L62fs		F1	+/p.L62fs	
NGN3	**11**	N5.4	g.395_410del/g.395_410del	**5**			NGN3-cr5
		N5.31	g.395_410del/g.395_410del				
		N5.8	g.395_920del/g.395_920del ($NGN3^{\Delta/\Delta}$)				

N6.10	p.Q4fs/p.Q4fs	N6.30	+/p.Q4fs	NGN3-cr6
N6.22	p.Q4fs/p.Q4fs			
H5	p.L106fs/p.L106fs	A2	+/p.L106fs	NGN3-cr3
B12	p.R107fs/p.R107fs	A4	+/p.A105fs	
A10	p.G108fs/p.G108fs	C10	+/p.G108fs	
E7	p.G108fs/p.G108fs	D2	+/p.L106fs	NGN3-cr4
A6	p.R107S/p.R107S			
E11	p.R107S/p.R107S			

We examined a total of 62 biallelic and monoallelic hESC mutant lines affecting 8 individual genes. In most cases, at least two gRNAs were used to target distinct sequences in each gene, and 4 biallelic and 4 monoallelic mutants were chosen for further examination. Mutant alleles are described according to the predicted changes at the protein level following the Human Genome Variation Society (HGVS) guidelines (http://www.hgvs.org/mutnomen/). In brief, the first amino acid affected and its position is described. Following that, "fs" indicates a frameshift change, "*" indicates an immediate stop codon and an amino acid code indicates a substitution. The single-letter amino acid code is used for simplicity. When a start codon is disrupted, changes in the genomic DNA sequence is described instead.

[#]**:** The *ARX* gene is on the X chromosome. As the parental HUES8 line was from a male donor (Cowan et al., 2004), mutant lines with the -/Y genotype is placed in the "biallelic mutant" category.

Supplemental Table 4, related to Figure 2. Oligonucleotides used in this study

PCR genotyping primers for detection of correct transgene integration into the *AAVS1* locus

Primers	Sequence (5' to 3')
F	CTGCCGTCTCTCTCCTGAGT
Puro-R	GTGGGCTTGTACTCGGTCAT
Neo-R	CTCGTCCTGCAGTTCATTCA
Hygro-R	GACATATCCACGCCCTCCTA

Primers and ssDNAs for generating templates for gRNA *in vitro* transcription

Target locus	CRISPR	Synthetic ssDNA sequence (5' to 3')
PDX1	**cr3**	TAATACGACTCACTATAGGGGGCCCATGTACAGGCACGCAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTT
	cr6	TAATACGACTCACTATAGGGACAGCTGCTAGAGCTGGAGAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTT
PTF1A	**cr1**	TAATACGACTCACTATAGGGGCGCCGCCGCTCGCGCACGTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTT
	cr4	TAATACGACTCACTATAGGGCTTGGAGAGGCGCTTCTCGTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTT
MNX1	**cr1**	TAATACGACTCACTATAGGGTGGTGAAGGCGGTGCGCGGCGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTT
	cr3	TAATACGACTCACTATAGGGGTCGCGGCCCAAGCGCTTCGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTT
NGN3	**cr5**	TAATACGACTCACTATAGGGCTTCCCTCTGACCCCGCCGTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTT
	cr6	TAATACGACTCACTATAGGGTAGGATGACGCCTCAACCCTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTT
	cr3	TAATACGACTCACTATAGGGTCGGCACTGGACGCCCTGCGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTT
	cr4	TAATACGACTCACTATAGGGAGGTGGGCAGGACACCGCGCGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTT
NGN3 (correction of *Q4fs*)	**cr1-Cr**	TAATACGACTCACTATAGGGCCCGCCGTAGGATGACGCCTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTT
	cr2-Cr	TAATACGACTCACTATAGGGCCGCCGTAGGATGACGCCTCGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTT
	cr3-Cr	TAATACGACTCACTATAGGGGCACCCGAGGCGTCATCCTAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTT
GLIS3	**cr2**	TAATACGACTCACTATAGGGGCTCGTGCGGCACATCGAGAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTT
	cr6	TAATACGACTCACTATAGGGGTGGATCAGCAGTTTATAGCGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTT
RFX6	**cr2**	TAATACGACTCACTATAGGGTGTGCATAAAGAATGCACCGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTT
	cr5	TAATACGACTCACTATAGGGGCTCAACACCTTGTATACCAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTT
ARX	**cr2**	TAATACGACTCACTATAGGGCGGGTAGTGCGTCTTCTGGAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTT
	cr4	TAATACGACTCACTATAGGGCATACCTGGTGAAGACGTCCGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTT
HES1	**cr1**	TAATACGACTCACTATAGGGGCAGTCATCAAAGCCTATTAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTT
	cr4	TAATACGACTCACTATAGGGCCAGCTGAAAACACTGATTTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTT
AAVS1 (Cas9/NGN3 exchange)	**cr1-ex**	TAATACGACTCACTATAGGGGATGACCGAGTACAAGCCCAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTT
	cr2-ex	TAATACGACTCACTATAGGGGACAGTACTAAGCTTTACTAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTT
Universal PCR primers (for generating dsDNA template needed for gRNA *in vitro* transcription)		**T7-F:** TAATACGACTCACTATAGGG **gRNA-R:** AAAAGCACCGACTCGGTGCC

The 20-nt target sequence is highlighted in red.

ssDNA HDR template for precise nucleotide alterations at the *NGN3* locus

Purpose	Synthetic ssDNA sequence (5' to 3')
To introduce the *NGN3 c.319C>A* (*p. R107S*) mutation.	ACCGCGAGCGCAATCGAATGCACAACCTCAACTCGGCACTGGACGCCCTGAGCGGTGTCCTGCCCACCTTCCCAGACGACGCGAA GCTCACCAAGATCGA
To correct the *p.Q4fs* mutation in the N6.10 mutant line to wild-type sequence.	CCTCGGAATCGCGGACTGCGCCGTGACGGACTCAAACTTACCCTTCCCTCTGACCCCGCCGTAGGATGACGCCTCAGCCCTCGGG TGCGCCCACTGTCCAAGTGACCCGTGAGACGGAGCGGTCCTTCCCCAGAGC

The base substitution compared to the wild-type sequence is highlighted in red.

PCR primers for T7EI, RFLP and Sanger sequencing

Gene	CRISPR	Forward primer (5' to 3')	Reserve primer (5' to 3')	Sequencing primer (5' to 3')
PDX1	**cr3**	CTGGGCCTAGCCTCTTAGTG	TGAGCTTTGGTAGACTTCATCC	CTGTGGGTTCCCTCTGAGAT
	cr6	TGAACTACACAACGATCCGA	CACAAACAACGCCAATCCAG	GGCTTGAGTTACTAGGGAAGAG
PTF1A	**cr1 & cr4**	CGGCTACTGCTGCGAGAC	GCTCGCATTCAAGTTTTTCC	AGGCGGCTTCCCCTACTC
MNX1	**cr1 & cr3**	AGGAAGCCTCCTGCGATG	CCACCCGAAGCTACTGAATC	ATTTCCACTTGGTGGTCCTG
NGN3	**cr5 & cr6**	CGGACCCCATTCTCTCTTCT	CCGGGTAGTGCTACCATTCT	CTATTCTTTTGCGCCGGTAG
	cr3 & cr4	CGGACCCCATTCTCTCTTCT	CCGGGTAGTGCTACCATTCT	CCTTACCCTTAGCACCCACA
GLIS3	**cr2 & cr6**	GAGGTCTACGGGCATTTCCT	AACCCCATCTCATGGATACCT	GGCCTGTTCAAGACCGAAC
RFX6	**cr2**	GAGAATGTAAGCATTTGCCAACC	TAGCAAGAGACTTGTCACCTCC	TGGACCAGGCATGGTTTTAT
	cr5	GAAGGTTGGATTTGACAATTCCTG	GTTCTTGCATTCATCTAGCATGTC	GCAGATCTTCCTAAACTTATGCCA
ARX	**cr2 & cr4**	TGGAGGACGAAGAAGATGAG	CAGCCTAAACTTAAAGCCCGA	ATGAGGACGAGGAAGAGGAA
HES1	**cr1 & cr4**	CGGATAAACCAAAGACAGCA	CATAGAGTAGGCAAGAAAGGA	TGTATCTCTTTGCAGCCCCT

Primers for qRT-PCR

Gene	Forward primer (5' to 3')	Reserve primer (5' to 3')
***NotchIC* (transgene)**	ACCCACTCTGTCTCCCACAC	GCTTCCTTGCTACCACAAGC
***Ngn3* (transgene)**	CCCCAGAGACACAACAACCT	GCAGTCACCCACTTCTGCTT
GAPDH	GGAGCCAAACGGGTCATCATCTC	GAGGGGCCATCCACAGTCTTCT
ACTB	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT
PDX1	TGGAGCTGGCTGTCATGTTGA	CGCTTCTTGTCCTCCTCCTTTT
PTF1A	CCAGAAGGTCATCATCTGCC	AGAGAGTGTCCTGCTAGGGG
MNX1	CCCAGGTGAAGATTTGGTTC	TTCTGTTTCTCCGCTTCCTG
SOX9	AGCTCTGGAGACTTCTGAACGAGAG	CGTTCTTCACCGACTTCCTCCGC
NKX6.1	CTGGCCTGTACCCCTCATCA	CTTCCCGTCTTTGTCCAACAA
NGN3	CTATTCTTTTGCGCCGGTAG	ACTTCGTCTTCCGAGGCTCT
INS	CCCTGCAGAAGCGTGGCATT	CCATCTCTCTCGGTGCAGGA
GCG	AAGCATTTACTTTGTGGCTGGATT	TGATCTGGATTTCTCCTCTGTGTCT
SST	GATGCTGTCCTGCCGCCTCC	TGCCATAGCCGGGTTTGA
PPY	CAGAGCAGATGGCCCAGTAT	CAGCGTGTCCTCTTTGTGTC
GHRL	TGAACACCAGAGAGTCCAGCA	GCTTGGCTGGTGGCTTCTT
NEUROD	GGATGACGATCAAAAGCCCAA	GCGTCTTAGAATAGCAAGGCA

RFX6	GTCGATGCATGGCTTGGACT	TGGGCCATAGCTAGACGGTG
NKX2.2	ATGTAAACGTTCTGACAACT	TTCCATATTTGAGAAATGTTTGC
ISL1	TTCCCTATGTGTTGGTTGCGGC	CGCATTTGATCCCGTACAACCTGA
MAFA	TTCAGCAAGGAGGAGGTCAT	CGCCAGCTTCTCGTATTTCT
MAFB	TCAAGTTCGACGTGAAGAAGG	GTTCATCTGCTGGTAGTTGCT
HES1	AGCTCGCGGCATTCCAAG	AGCGGGTCACCTCGTTCA
SLC2A1	GATTGGCTCCTTCTCTGTGG	TCAAAGGACTTGCCCAGTTT
SLC2A2	CATGTGCCACACTCACACAA	ATCCAAACTGGAAGGAACCC
GCK	TGCAGATGCTGGACGACAG	GAACTCTGCCAGGATCTGCTCTA
G6PC2	TGGTATGTCATGGTAACCGC	CACTCCAAAGAAATGACCAGG
ABCC8	CTGCTAAACCGGATCATCCTAGCC	CGAGGAACACAGGTGTGACATAGG
PCSK1	CACAATGACTGCACGGAGAC	ACCAGGTGCTGCATATCTCG
PCSK2	TGCAAAGGCCAAGAGAAGAC	TTTCGGTCAAATCCTTCCTG
SLC30A8	GATGCTGCCCACCTCTTAATTGAC	CCAAGACCAGGATGGAAAAGATGA

Primers for detecting potential large deletions in *NGN3* homozygous mutants

Forward primer (5' to 3')		Reserve primer (5' to 3')	
q1F:	CTATTCTTTTGCGCCGGTAG	**q1R:**	ACTTCGTCTTCCGAGGCTCT
q2F:	CAATCGAATGCACAACCTCA	**q2R:**	AGTCAGCGCCCAGATGTAGT
F:	CGGACCCCATTCTCTCTTCT	**R:**	CCGGGTAGTGCTACCATTCT
L1:	AAGTCCCCTCCAGGACAGAT	**R1:**	AGCGCTGAGAGACCAAACAT
L2:	TGAGCTCGTGGTTGTCTTTG	**R2:**	CTAGCGCTTTCCCAGTTCAC
L3:	GCACGCTGTGGTAGTTCAAA	**R3:**	CTCCACCTTCTTTGCTCCTG
L4:	TCACCCCACCCTACAGTCTC	**R4:**	CTCATGCTGCACCAGTCCTA
L5:	GTGCTTAGCCAGGTCAGGAG	**R5:**	AGGTGGCAGTTTGATGTTCC

Supplemental Table 5, related to Figure 3. hESC differentiation into polyhormonal pancreatic β cells

Stage	Day	Media	Supplement				
DE (3 days)	d0	Rinse cells with 1X DPBS w/ Mg^{2+}/Ca^{2+}					
		A-RPMI	FBS 0%	Activin A 100 ng/ml	BIO-acetoxime 2 μM		
	d1	A-RPMI	FBS 0.2%	Activin A 100 ng/ml			
	d3	Cells were examined for the expression of SOX17, FOXA2 and CXCR4.					
PP (6 days)	d3	A-RPMI	FBS 2%	FGF10 50 ng/ml	SANT-1 0.25 μM		
	d5	DMEM	B27 1%	FGF10 50 ng/ml	SANT-1 0.25 μM	RA 2 μM	LDN 250 nM
	d7	DMEM	B27 1%	FGF10 50 ng/ml	SANT-1 0.25 μM	RA 2 μM	LDN 250 nM
	d9	Cells were examined for the expression of PDX1 as well as other pancreatic progenitor markers.					
PH-β (8 days)	d9	DMEM	B27 1%	ALK5i II 1 μM	Noggin 100 ng/ml	DAPT 1 μM	
	d11	DMEM	B27 1%	ALK5i II 1 μM	Noggin 100 ng/ml	DAPT 1 μM	
	d13	DMEM	B27 1%				
	d15	DMEM	B27 1%				
	d17	Cells were examined for the expression of insulin and other endocrine markers.					

DMEM (high glucose) was purchased from the MSKCC media core. 1% Pen/Strep and 1% Glutamax were added into all media, and 0.005% BSA (bovine serum albumin) was added into all serum-free media. Media was changed on the indicated days.

Chemicals

Components	Vendor	Cat.
A-RPMI (Advanced RPMI)	Life Technologies	12633020
Activin A	PeproTech	120-14E
BIO-acetoxime, glycogen synthase kinase 3 (GSK-3) inhibitor	Tocris Bioscience	3874
FGF10	R&D Systems	345-FG-025
SANT-1, Hedgehog inhibitor	Tocris Bioscience	1974
RA (retinoic acid)	Sigma-Aldrich	R2625
LDN, BMP inhibitor	Stemgent	04-0019
ALK5i II (ALK5 inhibitor II)	Enzo Life Sciences	ALX-270-445
Noggin	PeproTech	250-38
DAPT, gamma-secretase inhibitor	Tocris Bioscience	2634

Supplemental Table 6, related to Figure 6. hESC differentiation into glucose-responsive β-like cells

Stage	Day	Media	Supplement							
S1 (3 days)	d0	Rinse cells with 1X DPBS w/o Mg^{2+}/Ca^{2+}								
		S1 media	GDF8 100 ng/ml	CHIR-99021 3 μM						
	d1	S1 media	GDF8 100 ng/ml	CHIR-99021 0. 3 μM						
	d2	S1 media	GDF8 100 ng/ml							
	d3	Cells were examined for the expression of SOX17, FOXA2 and CXCR4.								
S2 (2 days)	d3-d4	S1 media	L-Ascorbic Acid 0.25 mM	FGF7 50 ng/ml	IWP-2 1.25 μM					
S3 (2 days)	d5-d6	S3 media	L-Ascorbic Acid 0.25 mM	FGF7 50 ng/ml	SANT-1 0.25 μM	RA 1 μM	LDN 100 nM	TPB 200 nM	ITS-X 1:200	
S4 (3 days)	d7-d9	S3 media	L-Ascorbic Acid 0.25 mM	FGF7 2 ng/ml	SANT-1 0.25 μM	RA 0.1 μM	LDN 200 nM	TPB 100 nM	ITS-X 1:200	IWP-2 1.25 μM
	d10	Cells were examined for the expression of PDX1 and NKX6.1.								
S5 (3 days)	d10	S4 cells were treated with 10 μM Y-27632 for 4 hr and then dissociated into single cells using TrypLE Select (1X). Cell pellet was re-suspended in S5 medium at ~0.5 million cells/10μl and spotted on transwell (Corning) for culture in air-liquid interface. In generally, 5-10 μl/spot and 5 -10 spots were added in one 6-well transwell and 1.5 ml/well medium was added to the bottom of each insert in 6-well.								
	d10-d12	S5 media	3,3',5-Triiodo(T3) 1 μM	ALK5i II 10 μM	SANT-1 0.25 μM	RA 0.05 μM	LDN 100 nM	ITS-X 1:200	ZnSO4 10 μM	Heparin 10 μg/ml
S6 (7 days)	d13- d19	S5 media	3,3',5-Triiodo(T3) 1 μM	ALK5i II 10 μM	GSiXX 100 nM		LDN 100 nM	ITS-X 1:200	ZnSO4 10 μM	Heparin 10 μg/ml
S7 (7-14 days)	d20- d33	S5 media	3,3',5-Triiodo(T3) 1 μM	ALK5i II 10 μM	N-Cys 1mM	Trolox 10 μM	R428 2 μM	ITS-X 1:200	ZnSO4 10 μM	Heparin 10 μg/ml

Media was changed every day as indicated.

Media

S1 media	MCDB 131 + 1X Glutamax + 0.5% BSA + 1.5 g/l NaHCO3 + 10 mM Glucose
S3 media	MCDB 131 + 1X Glutamax + 2% BSA + 2.5 g/l NaHCO3 + 10 mM Glucose
S5 media	BLAR* + 1X Glutamax + 2% BSA + 1.5 g/l NaHCO3 + 20 mM Glucose

* BLAR was custom-made by Life Technologies with a published formulation (Rezania et al., 2014).

Chemicals

Components	Vendor	Cat.
MCDB 131	Life Technologies	10372-019
NaHCO3	Fisher Scientific	144-55-8
Glucose	Sigma-Aldrich	G8769
BSA	LAMPIRE Biological Laboratories	7500855
GDF8	PeproTech	120-00

CHIR-99021, GSK-3 inhibitor	Stemgent	04-0004
L-Ascorbic acid (vitamin C)	Sigma-Aldrich	A4544
FGF7	R&D Systems	251-KG
SANT1, Hedgehog inhibitor	Tocris Bioscience	1974
RA (retinoic acid)	Sigma-Aldrich	R2625
LDN, BMP inhibitor	Stemgent	04-0019
IWP-2, Wnt antagonist	Tocris Bioscience	3533
ITS-X	Life Technologies	51500-056
TPB, PKC activator	Provided by Alireza Rezania at Betalogics	
3,3',5-Triiodo-L-thyronine (T3)	Sigma-Aldrich	T6397
ALK5i II (ALK5 inhibitor II)	Enzo Life Sciences	ALX-270-445
ZnSO4	Sigma-Aldrich	Z0251
Heparin	Sigma-Aldrich	H3149
GSiXX (gamma secretase inhibitor XX)	EMD Millipore	565789
N-Cys (N-acetyl cysteine)	Sigma-Aldrich	A9165
Trolox, vitamin E analogue	EMD Millipore	648471
R428, AXL receptor tyrosine kinase inhibitor	Selleck Chemicals	S2841

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